

## Increased EBV Shedding in Astronaut Saliva During Spaceflight

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## **Abstract**

Shedding of Epstein-Barr virus (EBV) by astronauts before, during, and after space shuttle missions was quantified. Of 1398 saliva specimens from 32 astronauts, 314 (23%) were positive for EBV DNA by PCR analysis. Of the saliva specimens collected before flight, 29% were positive for EBV DNA and of those collected during or after flight, 16% were EBV-positive. The number of EBV DNA copies from samples taken during the flights was  $417 \pm 31$ , significantly higher ( $P < 0.05$ ) than the number of copies from the preflight ( $40 \pm 1.7$ ) and postflight ( $44 \pm 5$ ) phases. Eighteen control subjects shed EBV DNA with a frequency of 3.7% and a copy number of  $40 \pm 2$  per ml saliva. Ten days before flight and on landing day, antibody titers to EBV viral capsid antigen (VCA) were significantly ( $P < 0.05$ ) higher than baseline levels. On landing day, urinary levels of cortisol and catecholamines, and plasma levels of substance P and other neuropeptides, were increased over their preflight values. Results suggested that stress associated with spaceflight decreases cellular immunity and thereby leads to increased viral reactivation.

## **Introduction**

Spaceflight is a unique and stressful environment for humans. Astronauts experience a wide spectrum of stresses, such as isolation, confinement, fear, anxiety, psychosocial issues, sleep deprivation, and microgravity. These stressors may be intermittent or constant. Law enforcement officers, military personnel, and others also experience some of these stressors. However, microgravity is a unique and constant stress, making spaceflight an environment not duplicated anywhere on Earth. A wide range of physiological effects associated with spaceflight, such as bone loss, muscle atrophy, neurological anomalies, and cardiovascular

deconditioning, have been reported and reviewed [1-5] . The human immune response is also affected by spaceflight.[6] Changes have been reported in white blood cell numbers, leukocyte and lymphocyte subsets, T-cell proliferation, cytokine production, natural killer cell cytotoxicity, and cell-mediated immunity [7-11] .

Glaser and others [12] have shown that stress is associated with the onset, duration, and intensity of herpesvirus reactivation. We have previously shown that reactivation of Epstein-Barr virus (EBV), cytomegalovirus (CMV), and varicella-zoster virus (VZV) increases in astronauts participating in space shuttle missions [13-15] . Psychological stress has been implicated in the down-regulation of the immune system, and the response is mediated, in part, through the action of stress hormones. The effects are remarkably similar to those seen in astronauts after flight [16] . In a group of Antarctic expeditioners serving as a ground-based model for spaceflight, we showed that increased shedding of EBV was concomitant with decreased cellular immunity [17] . This is consistent with Glaser et al.'s [18] findings that antibody titers to the viral capsid antigen (VCA) of EBV increased with the down-regulation of the cellular immune response in a student stress model. The increased EBV-specific antibody titers resulted from the memory B lymphocytes' response to increased amounts of viral protein after reactivation. We observed similar increases in CMV antibodies in astronauts, beginning with the preflight phase and continuing during the flight and even during the first few days after landing [14]

Previous studies showed that the incidence of EBV reactivation in astronauts was higher during the preflight phase than either the inflight or postflight phase [13] . This contrasted with our findings in Antarctic expeditioners, where the incidence was higher in the 8- to 9-month winter-over isolation phase [17] . This study was undertaken to expand on our initial report of EBV



reactivation in astronauts [13] by determining the amount of EBV shed during the three (before, during, and after) phases of a space shuttle mission.

## **Materials and Methods**

Subjects were 32 astronauts (24 men aged 37 through 57 years, mean = 44 years; 8 women aged 32 through 47 years, mean = 39 years), all seropositive for EBV. They flew on 10 Space Shuttle missions of 9 to 14 days duration. Two Russian cosmonauts participating in an 83-day mission aboard the Russian space station Mir were also included in this study. Baseline (preflight) saliva samples were collected every other day for 1 to 2 months, beginning about 6 months before the flight. In-flight saliva samples were collected from crew members every day after their sleep period. Postflight samples were collected on landing day (R+0) and every day for 2 weeks thereafter. Samples were collected in 1.0 ml of a biocidal storage buffer (1% SDS, 10m M Tris-Cl, 1 mM EDTA) and stored at room temperature during the mission. Upon return of the samples to Earth, the saliva was collected by centrifugation and stored frozen until it was processed. All samples collected from each mission were analyzed simultaneously. Ground-based analysis verified the ability of the stability buffer to preserve the viral DNA for subsequent PCR analysis [13]

For measurement of viral antibody titers and stress hormone levels, a 10-ml EDTA blood sample and a urine sample were collected from each crew member 10 days before launch (L-10), 2 to 3 hours after landing (R+0), and 3 days after landing (R+3). Plasma was separated by centrifugation and stored at -70 °C until it was processed. All human study

protocols were approved by the Committee for the Protection of Human Subjects of the Johnson Space Center.

Controls: Eighteen healthy age-matched adults (14 male, 4 female) were included in this group. A saliva sample, a urine sample (10 ml), and a blood sample (10 ml, EDTA) were collected at 3 time points from all 18 subjects. The schedule of collection of these samples mimicked the spaceflight (12-day) sample collection schedule (day 0 = L-10, day 22 = R+0 for a 12-day flight, and day 25 = R+3. These samples were treated the same way as those of crew members to measure EBV DNA, antibody titer, and stress factors.

#### *Detection of EBV DNA*

Saliva specimens were concentrated with a 100K filtration unit (Filtron Technology Corp., Northborough, MA) and extracted by a nonorganic extraction method (Qiagen Inc., Chatsworth, CA). EBV was detected with Digene Diagnostics, Gaithersburg, MD as described earlier [13].

#### *Quantitative estimation of EBV*

EBV copies were measured in positive DNA samples by using the Viral Quant EBV quantitative PCR detection kit (Biosource International, CA). A known number of copies of exogenous synthesized DNA internal calibration standard (ICS) were mixed with sample DNA before it was extracted and amplified. The ICS has been constructed to contain a PCR primer binding site identical to that of EBV DNA and a unique capture



binding site that allows the resulting ICS amplicon to be distinguished from the viral amplicon. EBV DNA was amplified with Viral Quant format primers, one of which is biotinylated. These primers target a conserved sequence of EBER 1. The EBER1 gene is expressed during EBV latency as a small nonpolyadenylated RNA transcribed by RNA pol III. Sequences recognized by the two EBV-specific amplification primers are identical for the type 1 and type 2 strains of EBV. After PCR was performed, the amplicons were denatured and hybridized to either ICS or EBV sequence-specific capture oligonucleotides. Details are given elsewhere [19]

#### *Measurement of EBV antibody titer*

The titers of antibodies to EBV antigens VCA and early antigen (EA) were determined by indirect immunofluorescence assay (IFA) in the plasma samples collected from crew members 10 days before flight, at landing, and 3 days after landing. Titers of antibodies to EBV-VCA were also determined in samples archived from the annual physical examinations of the astronauts, which served as the baseline. These measurements were also carried out on the samples collected from 18 controls. Commercially-prepared substrate slides and control sera were used for determining EBV IgG and measles IgG antibody titers (Bion Enterprises, Park Ridge, IL). Tenfold dilutions of plasma were prepared with PBS. The endpoint titer was determined as the highest dilution of serum giving immunofluorescent cells. All specimens were batch analyzed and read blind-coded.

### *Stress hormones*

Plasma cortisol, adrenocorticotrophic hormone (ACTH), and human growth hormone (HGH), and urinary cortisol, norepinephrine, and epinephrine were measured by radioimmunoassay [20, 21] in the samples collected from astronauts before and after spaceflight.

### *Neuropeptides*

The neuropeptides substance P, calcitonin gene-related peptide, neuropeptide Y, and vasoactive intestinal peptide were measured in plasma from the 5 astronauts who participated in one of the 10 space shuttle flights included in the study. They were measured in samples collected 10 days before the flight, 2 to 3 hours after landing, and 3 days after landing by a receptor-affinity chromatographic technique coupled with immunological detection [22]

### *Statistical analysis*

To test whether EBV activation was significantly increased during flight we first expressed each subject's in-flight EBV copies as a difference from his/her pre-flight average. Next, this change in EBV copies was regressed on time in flight (days) using generalized estimating equations in a general linear model setting [23] with a normal family. In the process, standard errors were obtained using the Huber-White "sandwich" estimator [24, 25] to account for repeated measurements on some subjects at different times during flight; for example, one subject's EBV count was measured on nine different days. After fitting the regression model, we tested the null hypothesis of no increased activation any time during flight, which is equivalent to both the



intercept and the slope of the regression line being zero. Confidence limits for both the slope and intercept were also obtained.

## Results

EBV DNA was detected in 22.5% of the saliva samples (314/1398) collected from 32 astronauts before, during, or after 10 spaceflights (Table 1). This rate was significantly higher ( $P < 0.05$ ) than the 3.7% EBV-positive samples from the 18 control subjects. Of the saliva specimens collected about 6 months before flight, 29.1% were positive for EBV DNA. Of those collected during and after spaceflight EBV was positive in 15.7% and 15.5% samples respectively. Control values over a period simulating a 12-day space shuttle mission did not vary significantly.

The distribution of EBV copies found before, during, and after the flight is shown in Figure 1. Although the frequency of EBV DNA was nearly 2-fold greater in the samples collected before flight than in those collected at other times, the number of copies of EBV DNA was significantly ( $P < 0.05$ ) higher (about 10-fold) in the samples collected during flight than in preflight or postflight samples. The number of EBV copies (mean per ml saliva  $\pm$  SE) was  $40.3 \pm 1.7$  before,  $417.2 \pm 31.1$  during, and  $44.3 \pm 5.4$  after flight. The median of the number of EBV copies detected in saliva samples increased as the duration of flight increased. The estimated equation for  $Y$ , (the increase in EBV count) is  $Y = 23.9t + 215$ , where  $t$  is flight time in days. The test of the null hypothesis of no increase at any time during flight was overwhelmingly rejected ( $P < .00001$ ), that is, there was a significant increase in EBV copies during flight than pre or post flight. In addition the rate of increased EBV copies per day of flight (23.9) was significantly greater than zero ( $P = 0.00013$ ) that is, as the duration of flight

increases, EBV copies increase significantly. Ninety-five percent confidence limits for the daily increase were 11.6 and 36.1.

The durations of these space shuttle missions ranged from 9 to 14 days. Two Russian cosmonauts participating in an 83-day mission aboard the Russian space station Mir had similar EBV shedding patterns and an increased number of EBV copies during the flight phase. The mean number of EBV copies per ml of saliva was  $24.7 \pm 3.9$  before the flight and  $18.8 \pm 6.9$  after the flight. During the long flight the number of EBV copies soared to  $451 \pm 79.3$ . These data, shown in Figure 2, were divided into three groups, first group, 21 to 40 days; second group, 41 to 60 days; and third, 61 to 75 days. The number of EBV copies was highest during the first and the third group during the flight.

Shuttle astronaut VCA antibody titers at 10 days before launch (L-10), at landing (R+0), and 3 days after landing (R+3) were significantly increased ( $P < 0.001$ ) from the baseline values taken 5 to 24 months before flight (Figure 3). Titers were the same at L-10 and R+0, but increased further at R+3 ( $P < 0.001$ ). As expected, no significant change was observed in the titer of antibodies to EBV early antigen (EA). As a control, we measured measles IgG antibody titer and found no change from baseline levels throughout the study interval. The VCA IgG antibody titers of 18 control subjects were similar to the astronaut baseline levels and did not change across the 3 sampling times.

Stress hormones were measured at three time points, one before launch (L-10) and two after landing (R+0 and R+3) (Figure 4). The level of cortisol in plasma was not significantly lower after landing than before flight, but plasma levels of insulin and



aldosterone increased (Panel A). Plasma ACTH and HGH did not change. Levels of cortisol, epinephrine, and norepinephrine in urine at R+0 were significantly higher than at L-10 (Panel B) ( $p < 0.05$ ). The controls had no such changes during the study period.

Neuropeptides were measured in plasma of 5 crew members before and after a 5-day flight. Levels of substance P (SP), calcitonin gene-related peptide (CGRP), neuropeptide Y (NY), and vasoactive intestinal peptide (VIP) were significantly higher ( $p < 0.05$ ) (Figure 4) at R+0 than at L-10. However, 3 days after landing, only SP showed a further increase which was statistically insignificant. CGRP, NY, and VIP either showed no further increase from their R+0 levels or declined. In case of control group, these changes were not observed.

## **Discussion**

This study is a follow-up of our initial finding that astronauts shed EBV before, during, and immediately after spaceflight [13]. We previously found that the shedding frequency was higher during the preflight phase than during either the flight or postflight phase. The current study confirmed that finding. The major objective was to determine if the number of copies of viral DNA correlated with shedding frequency. We quantified the amount of EBV shed in astronauts' saliva during all three phases of space shuttle missions. Although the frequency of EBV shedding in saliva was higher before flight, the number of EBV copies was 10-fold higher during the flight phase. This indicates that the intensity or combination of stressors experienced by astronauts while they are in space may be responsible for the increased EBV DNA in saliva. The data (Figures. 1 and 2) also indicated that the number of EBV copies increases as a function of days in space. Saliva

samples from cosmonauts aboard the Russian space station Mir for 83 days contained more copies of EBV DNA than saliva samples from the US astronauts particularly in space flight missions of 14 days or less. Also, the peak number of EBV copies (1130/ml) in samples from cosmonauts was higher than the maximum number of copies (738/ml) in samples from US astronauts. Taken together, data from the short-term and long-term space missions indicate that the numbers of EBV shed in saliva may increase with increasing time in space.

Glaser [18, 26, 27] and others have studied EBV reactivation in several stress models and found increased reactivation of EBV as measured by increases in antibody titers to VCA. Glaser et al. [12, 28] also showed that reactivation of EBV was a function of the type of stress. For example, physical stress associated with basic training of West Point cadets did not result in EBV reactivation. However, stress associated with final examinations resulted in substantial viral reactivation. Consistent with their work, we have found that VCA titers increase before flight and continue to increase through 3 days after flight. This indicates that astronauts are exposed to substantial levels of various stressors, resulting in EBV reactivation beginning well before flight.

Examining hormone profiles provided further evidence of stress exposure. Urinary levels of cortisol and the catecholamines were significantly higher after flight than before flight. These data were consistent with results from the Skylab missions [29] and previous space shuttle missions [14, 30]. Cortisol, epinephrine, and norepinephrine in urine are usually increased at landing, probably as a consequence of the stresses associated with the reentry



and landing process. Plasma levels of cortisol at landing were not elevated over preflight values. The peak plasma level of cortisol, which has a circadian rhythm, may have occurred before or after the sample was collected. Plasma cortisol, which has a short (~4-hour) half-life, was collected only once before and once after spaceflight, giving a “snapshot” look at plasma levels, whereas each urinary cortisol value was obtained from a pool of urine collected over a 24-hour period. The “snapshot” of plasma cortisol may have missed transient but significant changes that were detected in urine, which provided an integration of 24 hours of collection[30] . All of the neuropeptides measured (SP, VIP, NY, and CGRP), were elevated immediately after landing. Only SP had increased even more by 3 days after landing. Increased VIP, SP, and CGRP levels have been associated with decreased cellular immunity [31, 32] . Similarly, increased levels of NY have been found in chronic stress subjects [33] . Until the neuropeptide data from the 5 astronauts can be expanded to a larger number of participants, the significance of changes in neuropeptide levels cannot be adequately assessed. However, these findings are consistent with data from Antarctic expeditioners and astronauts. We previously demonstrated decreased cellular immunity in Antarctic expeditioners during a stressful 8-9 month winter-over period at the Australian Antarctic science stations [17] . Taylor et al. [6] demonstrated decreased cell-mediated immunity in astronauts during spaceflight.

The viral shedding patterns, virus-specific antibody titer response, increases in stress hormones, and increased levels of neuropeptides lead us to draw some conclusions. No correlation was observed between increased viral reactivation and age, sex, flight experience or nature of the duties (e.g., pilot vs. non pilot) of the crew members.

Astronauts are exposed to multiple stressors capable of reactivating EBV before, during,

and after flight aboard the space shuttle. The intensity or combination of stressors during the flight may vary significantly from the stressors experienced before or after the flight. Our data indicate that more EBV is shed in saliva during spaceflight than during the ground phases of preparing for the mission and recovering from spaceflight-associated stress. The increased amount of EBV DNA in saliva, coupled with the propensity of large and small saliva droplets to float in the microgravity of the crew compartment, may lead to increased risk of cross infection among crew members. One would expect minimal medical effects of such events in healthy individuals, but the effectiveness of the immune response of astronauts has been questioned [6] .

Studies are in progress to determine if the increased reactivation of EBV in astronauts is a general effect extending to other human herpes viruses, and if it produces any health effects in astronauts on space missions longer than 14 days.

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Figure 1

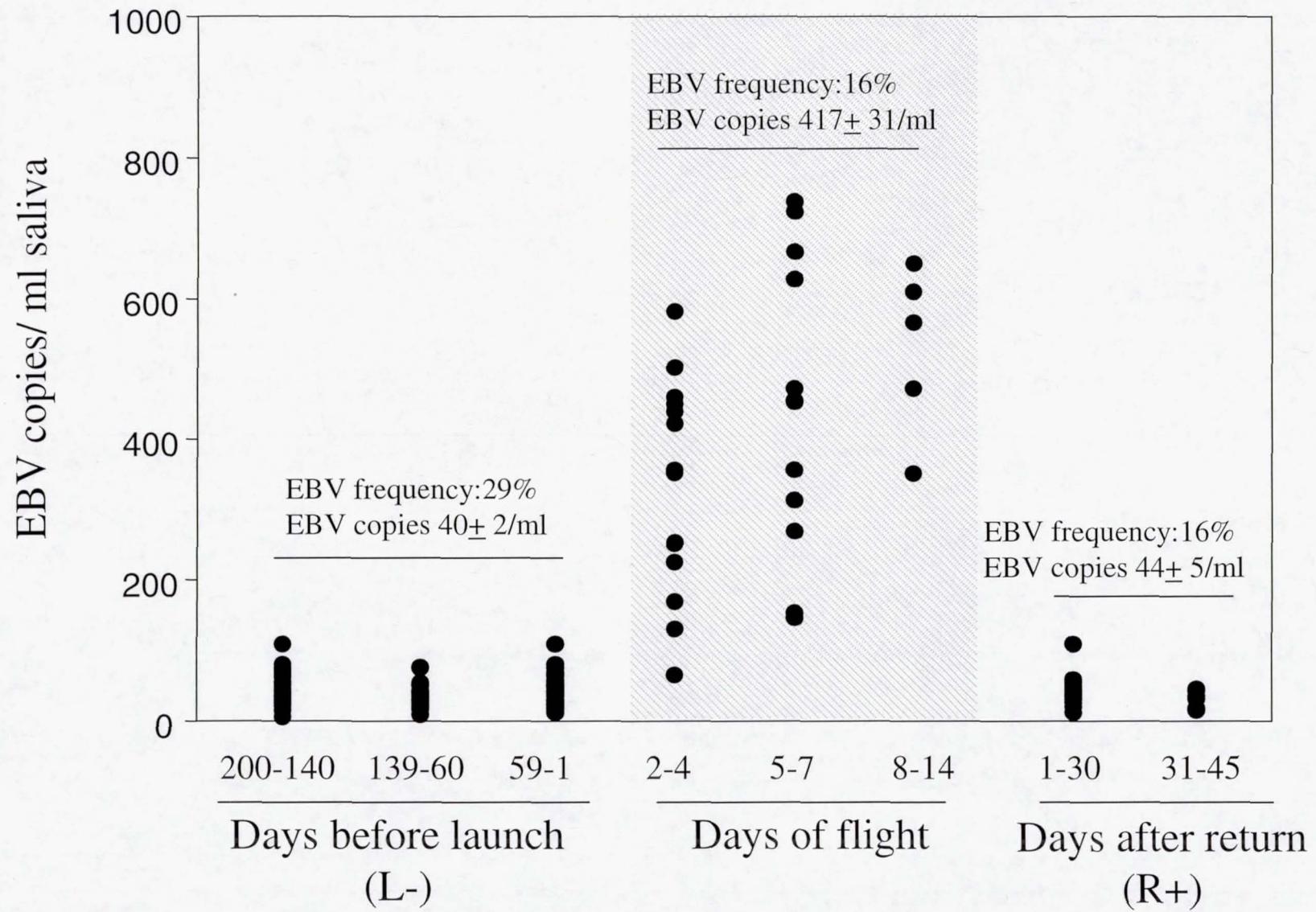




Figure 2

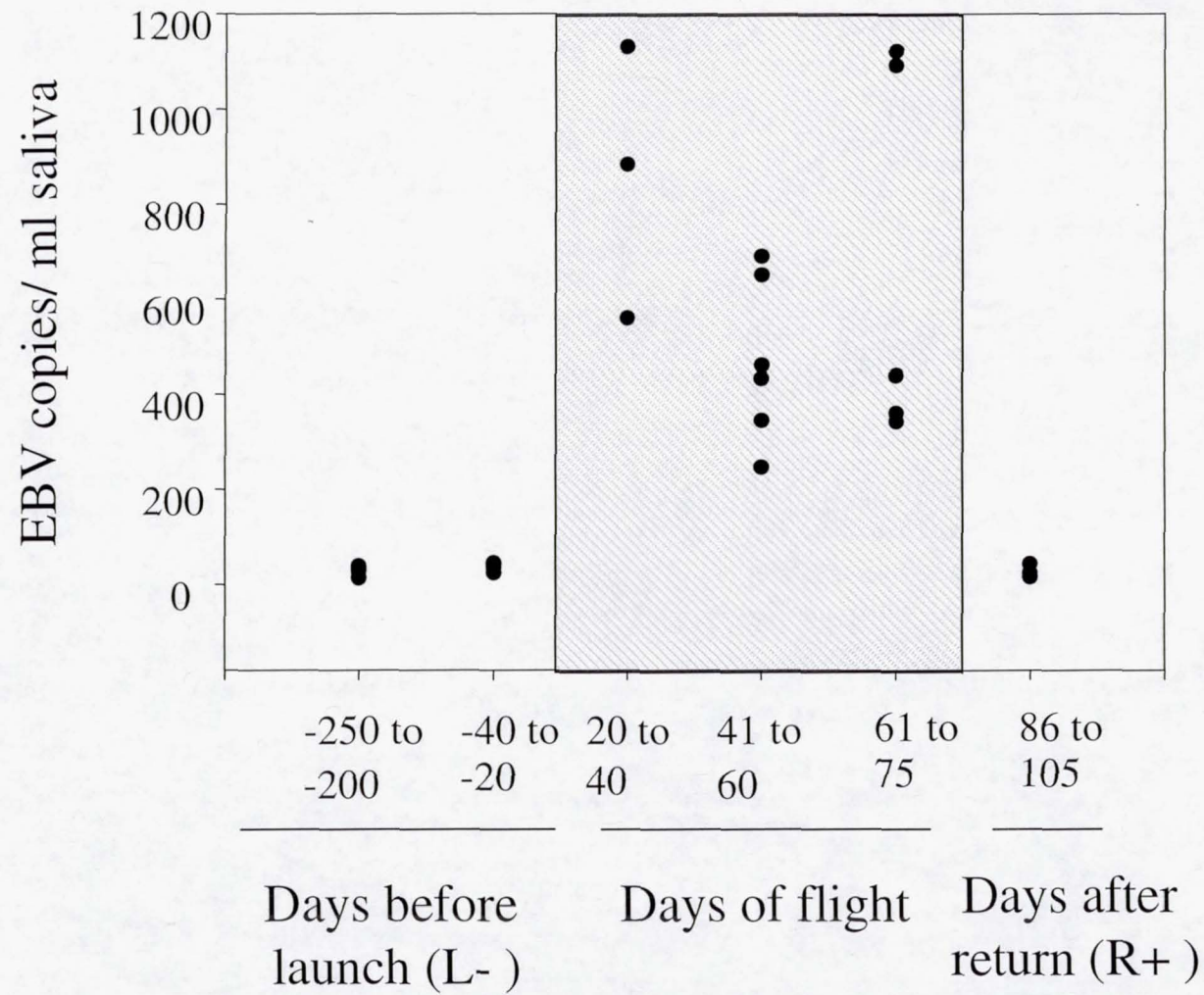


Figure 3

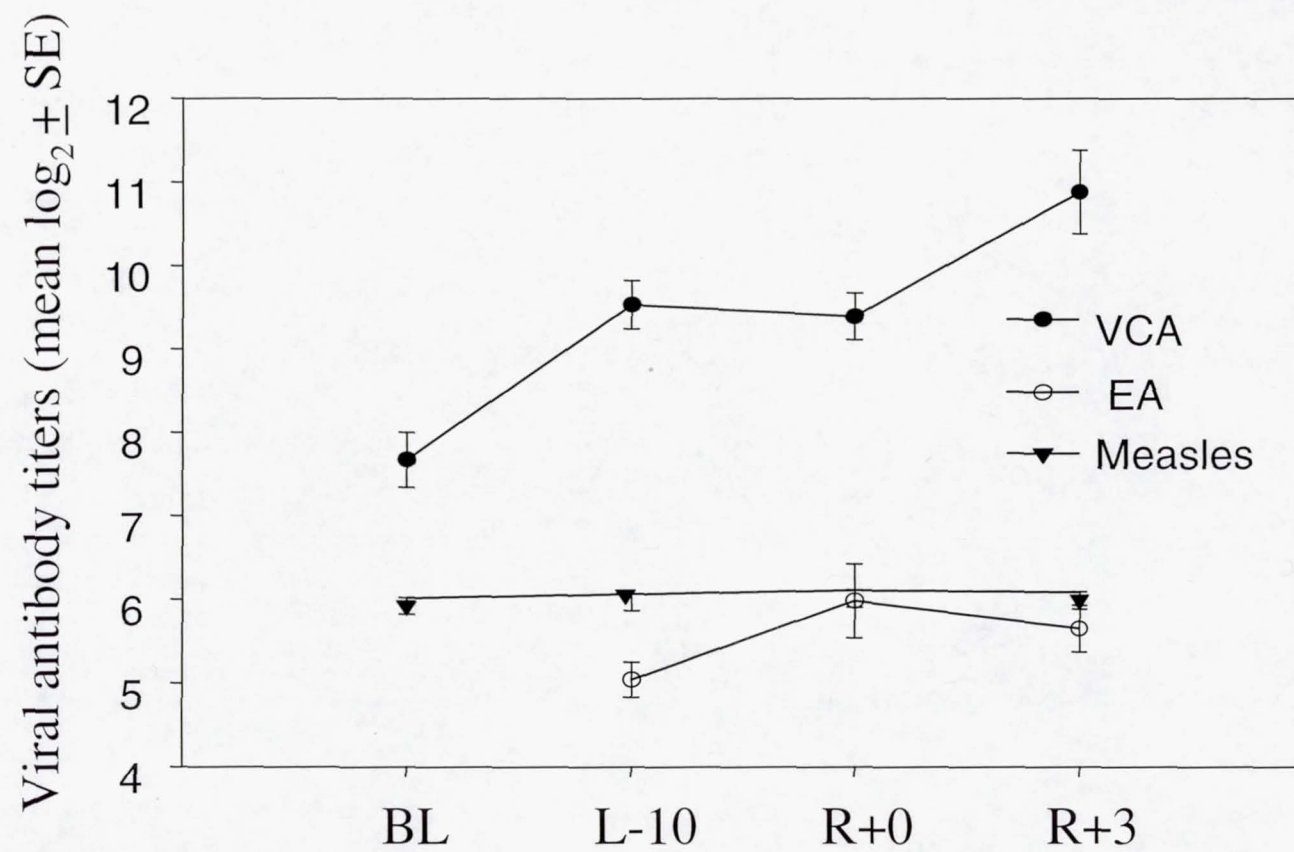
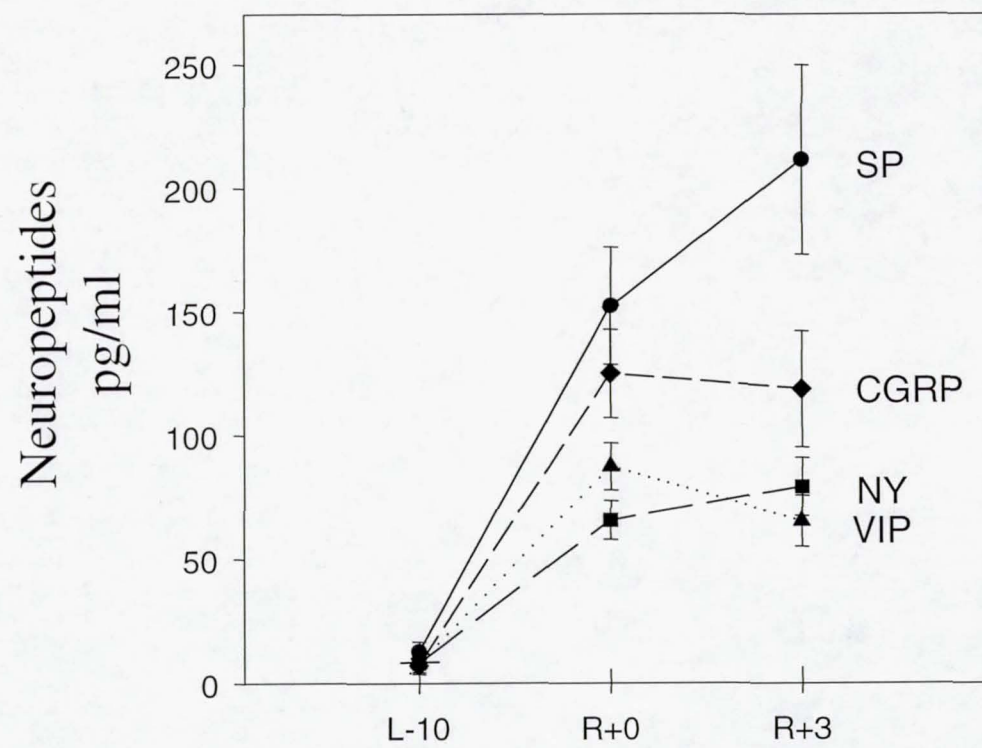




Figure 4



# Figure 5

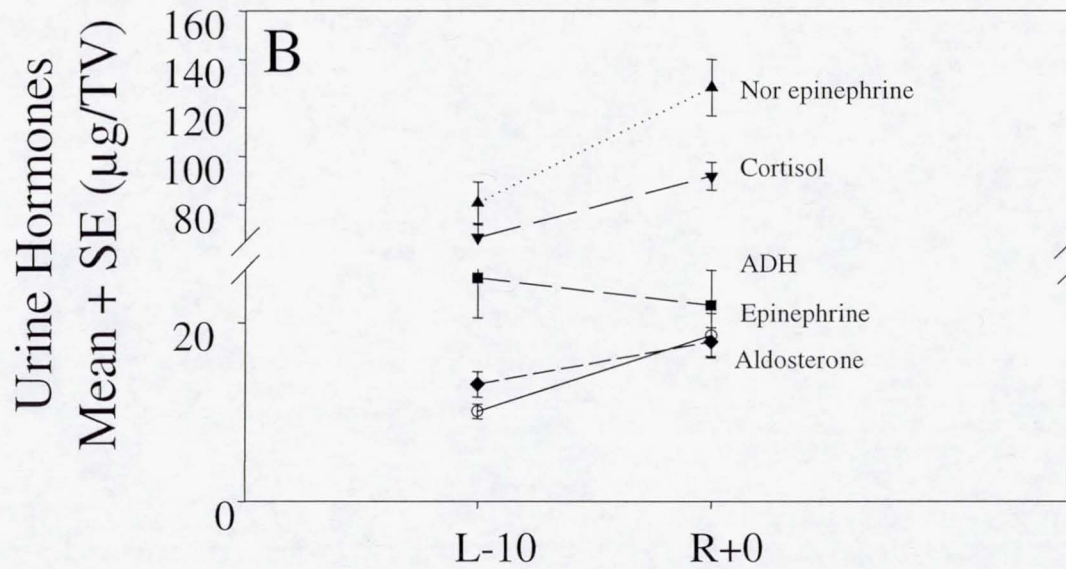
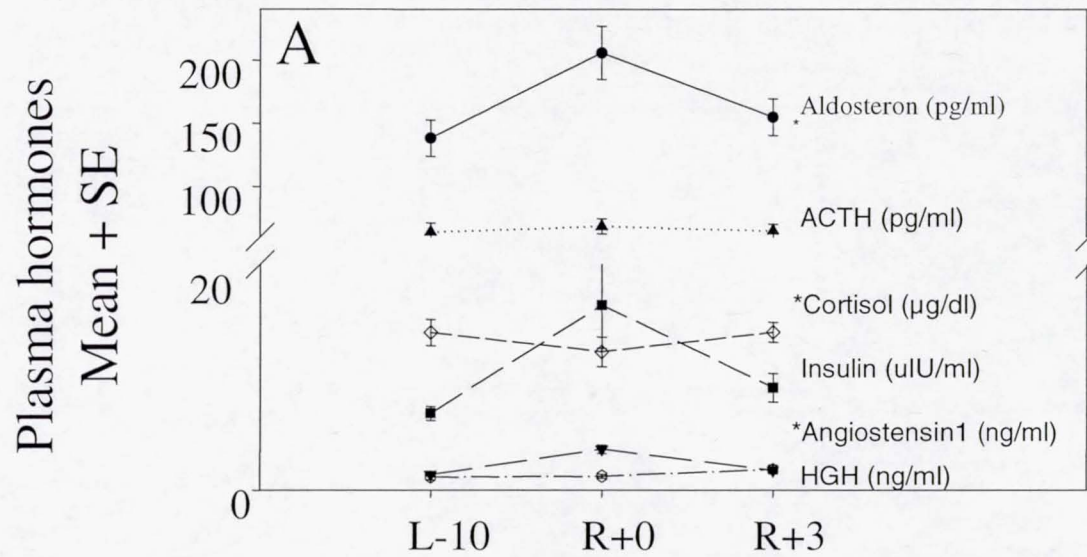




Figure 1. EBV copies/ml saliva in 32 astronauts before, during, and after 10 space shuttle missions.

Figure 2. EBV copies/ml saliva in crew members before, during, and after an 83-day mission aboard the Russian space station Mir .

Figure 3. Viral IgG antibody titers ( $\log_2$  mean  $\pm$  SE) in astronauts at Baseline (BL), 10 days before launch (L-10), at landing (R+0), and 3 days after landing (R+3). EA titers were not available at BL.

Figure 4. Stress hormones measured in plasma (panel A) and urine (panel B) in the 32 astronauts before and after space flights

Figure 5. Neuropeptides (mean  $\pm$  SE) in astronauts before and after a 5-day mission 10 days before launch (L-10), at landing (R+0), and 3 days after landing (R+3).